



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 5/04, 5/10, 5/16, 5/22, 15/82, A01H 5/00</b>		A1	(11) International Publication Number: <b>WO 99/02655</b>
			(43) International Publication Date: 21 January 1999 (21.01.99)
(21) International Application Number: PCT/US98/14109 (22) International Filing Date: 7 July 1998 (07.07.98) (30) Priority Data: 08/889,655                      8 July 1997 (08.07.97)                      US (71) Applicant (for all designated States except US): UNIVERSITY OF KENTUCKY RESEARCH FOUNDATION [US/US]; 207 Administration Building, Lexington, KY 40506-0032 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): LUSSO, Marcos, Fernando, Godoy [BR/US]; 2020 Armstrong Mill Road #2004, Lexington, KY 40515 (US). CHAPPELL, Joseph [US/US]; 1808 Bimini Road, Lexington, KY 40502 (US). (74) Agent: ELLINGER, Mark, S.; Fish & Richardson P.C., P.A., Suite 3300, 60th South 6th Street, Minneapolis, MN 55402 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GR, HU, IL, IN, JP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  Published With international search report.	
(54) Title: PROTEIN KINASES AND USES THEREOF			
(57) Abstract			
<p>Nucleic acid molecules are disclosed that are induced upon pathogen invasion or elicitor treatment. Such molecules are functional in plants, plant tissue and in plant cells for inducible gene expression and altering the disease resistance phenotype of plants. Such molecules are, or are related to, sequences of calcium dependent protein kinase genes. Also disclosed are methods for obtaining transgenic plants containing such nucleic acid molecules and methods for using such molecules. Polypeptides encoded by such nucleic acids are also disclosed herein.</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## PROTEIN KINASES AND USES THEREOF

### Statement as to Federally Sponsored Research

5       The research reported herein was performed in part with funding from the National Science Foundation of the United States Government. The United States Government may have certain rights in this invention.

### Field of the Invention

10       This invention relates to nucleic acids encoding calcium dependent protein kinases, polypeptides produced from such nucleic acids and transgenic plants expressing such nucleic acids.

### Background of the Invention

15       In plants, disease resistance to fungal, bacterial, and viral pathogens is associated with a plant response termed the hypersensitivity response (HR). In the HR, the site in the plant where the potential phytopathogen invades undergoes localized cell death, and  
20 it is postulated that this localized plant cell death contains the invading microorganism or virus, thereby protecting the remainder of the plant. Other plant defense responses include the production of phytoalexins, the production of lytic enzymes capable of averting  
25 pathogen ingress and modifications to cell walls that strengthen it against physical and/or enzymatic attack.

      The HR of plants can include phytoalexin production as part of the response to invading microorganisms. For example, tobacco (*Nicotiana tabacum*)  
30 produces sesquiterpenes in response to microbial invaders, e.g., *Pseudomonas lachrymans*.

      A variety of compositions can serve as elicitors of plant phytoalexin synthesis. These include one or

- 2 -

more toxic ions, e.g., mercuric ions, other chemically defined compositions, metabolic inhibitors, cell wall glycans, certain glycoproteins, certain enzymes, fungal spores, chitosans, certain fatty acids, and certain  
5 oligosaccharides derived from plant cell walls. See, e.g., Sequeira, L. (1983) *Annu. Rev. Microbiol.* 37:51-79 and references cited therein. Cell wall fragments of certain *Phytophthora* species and cellulase from *Trichoderma viride* but not *Aspergillus japonicum*  
10 pectolyase can also elicit the HR. Attack by other plant pathogens or an avirulent related strain can also induce the HR.

Elicitins are proteins produced by plant pathogens and potential plant pathogens. Elicitins can induce the  
15 HR in plants. Generally, but not necessarily, localized cell death is the result of the elicitin-induced response in the infected (or challenged) plant tissue. These responses mediate full or partial resistance to destructive infection by the invading, potentially plant  
20 pathogenic microorganism. Amino acid and nucleotide coding sequences for an elicitin of *Phytophthora parasitica* have been published. Kamoun et al. (1993) *Mol. Plant-Microbe Interactions* 6:573-581.

Plant pathogenic viruses including, but not  
25 limited to, Tobacco Mosaic Virus (TMV), induce the HR in infected plants. Bacteria that infect plants also can induce HR and thereby disease resistance; representative bacteria eliciting HR include, e.g., *Xanthomonas* spp. and *Pseudomonas syringae*. Plant pathogenic fungi generally  
30 do not induce the HR response after attack on a host plant, e.g., *Phytophthora parasitica* and *Peronospora tabaci* on tobacco hosts, but can induce the HR after attack on a non-host plant.

The signal transduction mechanisms involved in  
35 expression of disease resistance are under investigation

- 3 -

and some of the genetic and biochemical features have been outlined. See, e.g., Staskawicz, B. et al., Science 268:661-667 (1995). However, many aspects of signal transduction pathways and the role of many specific  
5 components are not well understood.

There is a long felt need in the art for methods of protecting plants, particularly crop plants, from infection by plant pathogens. Especially important from the standpoint of economic and environmental concerns are  
10 biological or "natural" methods rather than those which depend on the application of chemicals to crop plants. There is also a need in the art for plant polynucleotide sequences for enhancing and/or improving disease resistance in plants.

15                   Summary of the Invention

Nucleic acids of the present invention are based on novel calcium dependent protein kinase (CDPK) genes and their corresponding proteins. Induction of expression of these novel CDPK genes is surprisingly  
20 rapid, i.e., mRNA transcription of such genes can be observed as soon as 30 minutes after elicitor-mediated induction of plant defense responses. Thus, the novel genes disclosed herein are among those genes that are most rapidly induced in response to signals indicating an  
25 invading plant pathogen.

An isolated polynucleotide is disclosed herein, that comprises the nucleotide sequence of SEQ ID NO:1 and its complement, and an RNA analog of SEQ ID NO:1 or its complement. Such a polynucleotide can also be a nucleic  
30 acid fragment of the above that is at least 20 nucleotides in length and that hybridizes under stringent conditions to genomic DNA encoding the polypeptide of Figure 3. The polynucleotide can comprise, for example,

- 4 -

nucleotides 1 to 170, nucleotides 160 to 560, or nucleotides 550 to 920 of Figure 2.

A nucleic acid construct as disclosed herein comprises a polynucleotide of the invention. In such a  
5 construct, a polynucleotide of the invention can be operably linked to one or more elements that regulate transcription of the polynucleotide, for example, a regulatory element induced in response to a plant pathogen such as a fungus (e.g., *Phytophthora*), a  
10 bacterium (e.g., *Pseudomonas*), or a virus (e.g., Tobacco Mosaic Virus) as described herein. In other embodiments, such induction is mediated by an elicitor (e.g., by fungal or bacterial elicitors).

Further aspects of the present invention are  
15 transgenic plant cells, plant tissues, and plants that have been genetically engineered to contain and express a polynucleotide of the invention, for example, a coding sequence, or an antisense sequence. The construct can further comprises a regulatory element operably linked to  
20 the polynucleotide, e.g., an inducible regulatory element. The plant can be a dicotyledonous plant, e.g., a member of the *Solanaceae* family such as *Nicotiana tabacum*. The plant can also be a monocotyledonous plant, a gymnosperm, or a conifer.

25 A transgenic plant is disclosed herein that contains a polynucleotide expressing a polypeptide having from about 250 to about 550 amino acids. The polypeptide comprises an amino acid sequence substantially identical to the amino acid sequence of Figure 3.

30 A method of using a polynucleotide is disclosed herein. The method comprises the step of hybridizing the polynucleotide discussed above to DNA or RNA from a plant. The method can further comprise the steps of identifying a segment of the plant DNA or RNA that has  
35 about 70% or greater sequence identity to the

- 5 -

polynucleotide, and the step of cloning at least a portion of the DNA or RNA segment. The cloned portion may further comprise DNA flanking the segment having 70% or greater sequence identity.

- 5           In another aspect, the invention features a method of altering disease resistance in a plant. The method comprises the steps of introducing a polynucleotide of the invention into a plant cell; and producing a plant containing the polynucleotide from the plant cell.
- 10 Expression of the polynucleotide alters disease resistance in the plant. For example, the nucleic acid construct may further comprise an inducible regulatory element operably linked to the polynucleotide and expression may be induced by the regulatory element upon
- 15 exposure of the plant to an elicitor or plant pathogen.

          In another aspect, the invention features an isolated polypeptide, having from about 250 to about 550 amino acids and comprising an amino acid sequence substantially identical to Figure 3.

- 20           An inducible regulatory element is a DNA sequence effective for regulating the expression of a polynucleotide that is operably linked to that regulatory element. For example, a CDPK gene product associated with a plant defense response (e.g., a hypersensitive
- 25 response) can be operably linked to a developmentally-regulated regulatory element. Also included in this term are regulatory elements that are sufficient to render gene expression inducible in response to disease-associated external signals or agents (e.g., pathogen- or
- 30 elicitor-induced signals or agents as described herein). Also included in this term are those regulatory elements flanking a novel CDPK gene and involved in rapid induction of transcription of such a novel gene. In general, defense response regulatory elements are located

- 6 -

5' to the coding region of a gene, but are not so limited.

By "tissue-specific" is meant capable of preferentially increasing expression of a gene product (e.g., an mRNA molecule or polypeptide) in one tissue (e.g., xylem tissue) as compared to another tissue (e.g., phloem). By "cell-specific" is meant capable of preferentially increasing expression of a gene product (e.g., an mRNA molecule or polypeptide) in one cell (e.g., a parenchyma cell) as compared to another cell (e.g., an epidermal cell).

A "pathogen" is an organism whose infection of, or association with, cells of viable plant tissue can result in a disease. An "elicitor" is any molecule that is capable of initiating a plant defense response. Examples of elicitors include, without limitation, one or more toxic ions, e.g., mercuric ions, other chemically defined compositions, metabolic inhibitors, cell wall glycans, certain glycoproteins, certain enzymes, fungal spores, chitosans, certain fatty acids, and certain oligosaccharides derived from plant cell walls, and elicitors (e.g., harpin, cryptogin, and parasiticein).

By "operably linked" is meant that two polynucleotides are connected in such a way as to permit the two polynucleotides to achieve a desired functional activity, for example, linking of an inducible regulatory sequence and a coding sequence to achieve gene expression when the appropriate inducer molecules are present.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described

- 7 -

below. All publications, patent applications, patents,  
and other references mentioned herein are incorporated by  
reference in their entirety. In case of conflict, the  
present specification, including definitions, will  
5 control. In addition, the materials, methods, and  
examples are illustrative only and not intended to be  
limiting.

Other features and advantages of the invention  
will be apparent from the following description of the  
10 preferred embodiments thereof, and from the claims.

#### Brief Description of the Drawings

Figure 1 is a representation of the nucleotide  
sequences of the primers FokinB and RecalIV.

Figure 2 is a representation of the DNA sequence  
15 (SEQ ID NO:1) of a partial cDNA clone isolated from a  
cell suspension culture derived from a tobacco cultivar  
KY14 explant, after growth in the presence of the  
elicitin parasiticein.

Figure 3 is a representation of the deduced amino  
20 acid sequence of the DNA sequence of Figure 2, using the  
standard one letter amino acid code.

Figure 4 is a schematic comparison of the amino  
acid sequence of Figure 3 to that of a soybean CDPK.

#### Detailed Description of the Invention

25 The present invention relates to isolated  
polynucleotides (nucleic acids) that are induced in plant  
cells in response to invasion by a potential plant  
pathogen and/or treatment with an elicitor or elicitor-  
mimicking chemical signals. Such nucleic acids typically  
30 encode a calcium dependent protein kinase (CDPK)  
polypeptide or CDPK-related polypeptide. Induction of  
the novel polynucleotides disclosed herein corresponds in  
time to that of plant defense response genes, whereas

- 8 -

other CDPK genes appear to be induced less rapidly. Induction of gene expression for such novel genes is more rapid than that of genes involved in developmentally regulated processes in plants, e.g., developmentally regulated processes such as floral development. Induction of the novel CDPK genes disclosed herein is also more rapid than that of many genes involved in responses to abiotic stress, such as salt or water stress.

10 A polynucleotide of the present invention can be in the form of RNA or in the form of DNA, including cDNA, synthetic DNA or genomic DNA. The DNA can be double-stranded or single-stranded and, if single-stranded, can be either a coding strand or non-coding strand. An RNA  
15 analog of SEQ ID NO:1 may be, for example, mRNA or a combination of ribo- and deoxyribonucleotides.

A polynucleotide of the invention can encode a polypeptide including an amino acid sequence substantially similar or identical to that of Figure 3.

20 In some embodiments, a polynucleotide may be a variant of the nucleic acid shown in SEQ ID NO:1, e.g., can have a different nucleotide sequence that, due to the degeneracy of the genetic code, encodes the same amino acid sequence as the polypeptide of Figure 3.

25 A polynucleotide of the invention can further include additional nucleic acid sequences. For example, a nucleic acid fragment encoding a secretory or leader amino acid sequence can be fused in-frame to the amino terminal end of a polypeptide comprising the amino acid  
30 sequence of Figure 3. Other nucleic acid fragments are known in the art that encode amino acid sequences useful for fusing in-frame to the CDPK polypeptides disclosed herein. See, e.g., U.S. 5,629,193. A polynucleotide can further include one or more regulatory elements operably  
35 linked to a CDPK polynucleotide disclosed herein.

- 9 -

The present invention also includes polynucleotides that selectively hybridize to a CDPK polynucleotide sequence disclosed herein. Hybridization may involve Southern analysis (Southern blotting), a method by which the presence of DNA sequences in a target nucleic acid mixture are identified by hybridization to a labeled oligonucleotide or DNA fragment probe. Southern analysis typically involves electrophoretic separation of DNA digests on agarose gels, denaturation of the DNA after electrophoretic separation, and transfer of the DNA to nitrocellulose, nylon, or another suitable membrane support for analysis with a radiolabeled, biotinylated, or enzyme-labeled probe as described in sections 9.37-9.52 of Sambrook et al., (1989) *Molecular Cloning*, second edition, Cold Spring Harbor Laboratory, Plainview, NY.

A polynucleotide can hybridize under moderate stringency conditions or under high stringency conditions to a polynucleotide disclosed herein. High stringency conditions are used to identify nucleic acids that have a high degree of homology or sequence identity to the probe. High stringency conditions can include the use of a denaturing agent such as formamide during hybridization, e.g., 50% formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, and 75 mM sodium citrate at 42°C. Another example is the use of 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS. Alternatively, low ionic strength and high temperature can be employed for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate (0.1X SSC); 0.1% sodium lauryl sulfate (SDS) at 65°C.

- 10 -

Moderate stringency conditions are hybridization conditions used to identify nucleic acids that have less homology or identity to the probe than do nucleic acids identified under high stringency conditions. Moderate stringency conditions can include the use of higher ionic strength and/or lower temperatures for washing of the hybridization membrane, compared to the ionic strength and temperatures used for high stringency hybridization. For example, a wash solution comprising 0.060 M NaCl/0.0060 M sodium citrate (4X SSC) and 0.1% sodium lauryl sulfate (SDS) can be used at 50°C, with a last wash in 1X SSC, at 65°C. Alternatively, a hybridization wash in 1X SSC at 37°C can be used.

Hybridization can also be done by Northern analysis (Northern blotting), a method used to identify RNAs that hybridize to a probe. The probe is labeled with a radioisotope such as  $^{32}\text{P}$ , by biotinylation or with an enzyme. The RNA to be analyzed can be electrophoretically separated on an agarose or polyacrylamide gel, transferred to nitrocellulose, nylon, or other suitable membrane, and hybridized with the probe, using standard techniques well known in the art such as those described in sections 7.39-7.52 of Sambrook et al., *supra*.

It is generally preferred that a probe of at least about 20 nucleotides in length be used, preferably at least about 50 nucleotides, more preferably at least about 100 nucleotides. If a relatively short probe is to be used, the nucleotide sequence of the probe preferably avoids regions conserved among plant CDPK genes (protein kinase domains and calcium-binding domains), to more readily distinguish the rapidly induced CDPK genes disclosed herein from more slowly induced CDPK genes, constitutive CDPK genes or low-level constitutive CDPK genes. Nevertheless, probes containing such conserved

- 11 -

regions can be used, provided that there are sufficient non-conserved regions present in the probe that are more specific for the novel polynucleotides disclosed herein.

A polynucleotide of the invention has at least  
5 about 70% sequence identity, preferably at least about 80% sequence identity, more preferably at least about 90% sequence identity to SEQ ID NO:1. Sequence identity can be determined, for example, by computer programs designed to perform single and multiple sequence alignments.  
10 Polynucleotides having at least about 70% nucleotide sequence identity to the polynucleotide of SEQ ID NO:1 are included in the invention and can be identified by hybridization under conditions of moderate stringency. Polynucleotides having at least about 80% sequence  
15 identity, or at least about 90% sequence identity, or at least about 95% sequence identity to the polynucleotide of SEQ ID NO:1 can be identified by high stringency hybridization.

A polynucleotide of the invention can be obtained  
20 by chemical synthesis, isolation and cloning from plant genomic DNA, or other means known to the art, including the use of polymerase chain reaction (PCR) technology carried out using oligonucleotides corresponding to portions of SEQ ID NO:1. PCR refers to a procedure or  
25 technique in which target nucleic acid is amplified in a manner similar to that described in U.S. Patent No. 4,683,195, incorporated herein by reference, and subsequent modifications of the procedure described therein. Generally, sequence information from the ends  
30 of the region of interest or beyond are employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific RNA sequences, specific DNA sequences from total genomic DNA,  
35 and cDNA transcribed from total cellular RNA,

- 12 -

bacteriophage or plasmid sequences, and the like.

Alternatively, it is contemplated that a cDNA library (in an expression vector) can be screened with CDPK-specific antibody prepared using peptide sequence(s) from

5 hydrophilic regions of the CDPK sequence of Figure 3 and technology known in the art.

The novel polynucleotides of the invention can be found in substantially all plants, including members of the *Leguminaceae* (e.g., soybean), members of the

10 *Solanaceae* (e.g., *N. tabacum*), members of the *Brassicaceae* family (e.g., *Arabidopsis thaliana*) and members of the *Graminaceae* (e.g., *Zea mays*). Preferably, polynucleotides of the invention are selected from the *Solanaceae* family.

15 In some embodiments, a polynucleotide of the invention is identified and isolated from a plant based on nucleotide sequence homology and on the rapid induction of expression after elicitor or pathogen treatment. For example, DNA:DNA hybridization under  
20 conditions of moderate to high stringency with a polynucleotide probe disclosed herein allows the identification of corresponding genes from other plant species. Use of a target nucleic acid (e.g., cDNA) prepared from a tissue shortly after induction of defense  
25 responses facilitates the isolation of the novel polynucleotides disclosed herein, because such polynucleotides typically are more rapidly induced than other CDPK genes.

A nucleic acid construct comprises a  
30 polynucleotide as disclosed herein, and typically is linked to another, different polynucleotide. For example, a full-length CDPK coding sequence can be operably fused in-frame to a nucleic acid fragment that encodes a leader sequence, secretory sequence or other

- 13 -

additional amino acid sequences that may be usefully linked to a polypeptide or peptide fragment.

In some embodiments, a nucleic acid construct includes a polynucleotide of the invention operably  
5 linked to at least one suitable regulatory sequence in sense or antisense orientation. Regulatory sequences typically do not themselves code for a gene product. Instead, regulatory sequences affect the expression level of the coding sequence. Examples of regulatory sequences  
10 are known in the art and include, without limitation, minimal promoters and promoters of genes induced in response to elicitors. Native regulatory sequences of the polynucleotides disclosed herein can be readily isolated by those skilled in the art and used in  
15 constructs of the invention. Other examples of suitable regulatory sequences include enhancers or enhancer-like elements, introns, 3' non-coding regions such as poly A sequences and other regulatory sequences discussed herein. Molecular biology techniques for preparing such  
20 chimeric genes are known in the art.

Polypeptides of the invention have from about 250 to about 550 amino acids, e.g., from about 300 amino acids to about 508 amino acids, or from about 308 amino acids to about 500 amino acids. A polypeptide of the  
25 invention typically contains protein kinase domains as well as calcium-binding site domains. Such domains include, for example, amino acids 2 to 7, 42 to 49, 191 to 202, 227 to 238, 264 to 274, and 297 to 307 of Figure 3.

30 The amino acid sequence of the polypeptide can include the deduced amino acid sequence of Fig. 3. In other embodiments, a polypeptide of the invention includes an amino acid sequence substantially identical to that of Fig. 3, e.g., about 80% or greater sequence  
35 identity, or about 90% or greater sequence identity, or

- 14 -

about 95% or greater sequence identity. Generally, conservative amino acid substitutions or substitutions of similar amino acids are tolerated without affecting protein function. Similar amino acids are those that are  
5 similar in size and/or charge properties. For example, isoleucine and valine are similar amino acids. Similarity between amino acid pairs has been assessed in the art in a number of ways. For example, Dayhoff et al. (1978) in *Atlas of Protein Sequence and Structure*, Vol.  
10 5, Suppl. 3, pp. 345-352, provides frequency tables for amino acid substitutions which can be employed as a measure of amino acid similarity. Protein kinase domains and calcium-binding site domains may be altered by conservative substitutions, but generally are retained  
15 without alterations in amino acid sequence.

An "isolated" polypeptide is expressed and produced in a manner or environment other than the manner or environment in which the polypeptide is naturally expressed and produced. For example, a polypeptide is  
20 isolated when expressed and produced in bacteria or fungi. Similarly, a polypeptide is isolated when a gene encoding it is operably linked to a chimeric regulatory element and expressed in a tissue or species where the polypeptide is not naturally expressed. In addition, a  
25 polypeptide is isolated when a gene encoding it is operably linked to a chimeric regulatory element and is expressed in a tissue where the polypeptide is naturally expressed, but at higher levels. A polypeptide of the invention can also be isolated by standard purification  
30 methods to obtain it in about 80% or greater purity, or about 90% or greater purity or about 95% or greater purity.

In some embodiments, a polypeptide of the invention is an analog or variant of a polypeptide  
35 including the deduced amino acid sequence of Fig. 3.

- 15 -

Such analogs or variants include, for example, naturally occurring allelic variants, non-naturally occurring allelic variants, deletion variants, and insertion variants, that do not substantially alter the function of  
5 the polypeptide.

A polypeptide of the invention may comprise the sequence shown in Fig. 3 as well as the flanking amino terminal and carboxy terminal sequences encoded by the same gene as that comprising the nucleotide sequence of  
10 SEQ ID NO:1. Alternatively, a chimeric polypeptide may be produced from a gene that links, in-frame, nucleotides from the 5' region of a first CDPK gene to nucleotides from the 3' region of a second CDPK gene, thereby forming a chimeric gene that encodes the chimeric polypeptide.  
15 An illustrative example of a chimeric CDPK polypeptide is a polypeptide expressed by a polynucleotide encoding amino acids 1 to 156 from the amino terminal region of a soybean CDPK gene (Fig. 4), followed by the amino acid sequence of Fig. 3, followed by amino acids 465 to 508  
20 from the carboxy terminal region of the same soybean CDPK gene, all of which are fused in-frame.

A transgenic plant of the invention contains a nucleic acid construct as described herein. Such a construct is introduced into a plant cell and at least  
25 one transgenic plant is obtained. Seeds produced by a transgenic plant can be grown and selfed (or outcrossed and selfed) to obtain plants homozygous for the construct. Seeds can be analyzed to identify those homozygotes having the desired expression of the  
30 construct. Transgenic plants may be entered into a breeding program, e.g., to increase seed, to introgress the novel construct into other lines or species, or for further selection of other desirable traits.  
Alternatively, transgenic plants may be obtained by

- 16 -

vegetative propagation of a transformed plant cell, for those species amenable to such techniques.

As used herein, a transgenic plant also refers to progeny of an initial transgenic plant. Progeny includes  
5 descendants of a particular plant or plant line, e.g., seeds developed on an instant plant. Progeny of an instant plant also includes seeds formed on  $F_1$ ,  $F_2$ ,  $F_3$ , and subsequent generation plants, or seeds formed on  $BC_1$ ,  $BC_2$ ,  $BC_3$ , and subsequent generation plants.

10 In some embodiments, a transgenic plant contains a construct that includes a polynucleotide of the invention operably linked in sense orientation to a suitable regulatory element, so that a sense mRNA is produced. If desired, a selectable marker gene can be incorporated  
15 into the construct in order to facilitate identification of transformed cells or tissues.

Inhibition of the novel CDPK genes in plants is also useful. For example, inhibition of CDPK gene expression shortly before harvest of a seed crop can  
20 permit plant pathogens to more readily invade plant vegetative tissues, thereby reducing the amount of plant biomass that interferes with mechanical harvesting of the seeds. Regulated inhibition of CDPK gene expression can be accomplished by operably linking, in antisense  
25 orientation, a polynucleotide of the invention to a suitable inducible regulatory sequence. See, e.g., U.S. Patent 5,453,566. One can achieve the same effect by cosuppression, i.e., expression in the sense orientation of the entire or partial coding sequence of a novel CDPK  
30 gene can suppress corresponding endogenous CDPK genes. See, e.g., WO 94/11516.

In some embodiments, a nucleic acid construct includes a polynucleotide disclosed herein, operably linked to a minimal promoter. Such a construct, when  
35 introduced into and expressed in a plant, can confer low

- 17 -

level constitutive expression of the polynucleotide, resulting in an enhanced systemic defense response by the plant. A minimal promoter contains the DNA sequence signals necessary for RNA polymerase binding and  
5 initiation of transcription. Generally, transcription directed by a minimal promoter is low and does not respond either positively or negatively to environmental or developmental signals in plant tissue. An exemplary minimal promoter suitable for use in plants is the  
10 truncated CaMV 35S promoter, which contains the region from -90 to +8 of the 35S transcription unit.

Transcriptional regulatory sequences can be used to control gene expression in suspension cultures. For example, the EAS4 promoter including the transcription  
15 initiation signals, the inducible transcription regulatory element and the transcription-enhancing element, can be used to mediate the inducible expression of the disclosed coding sequence in transgenic plants or suspension cell cultures. See U.S. Application Serial  
20 No. 08/577,483. When desired, expression of the coding sequence of interest is induced by the application of an elicitor or other inducing signal.

Transgenic techniques for use in the invention include, without limitation, *Agrobacterium*-mediated  
25 transformation, electroporation and particle gun transformation. Illustrative examples of transformation techniques are described in U.S. Patent 5,204,253, (particle gun) and U.S. Patent 5,188,958 (*Agrobacterium*). Transformation methods utilizing the Ti and Ri plasmids  
30 of *Agrobacterium spp.* typically use binary type vectors. Walkerpeach, C. et al., in Plant Molecular Biology Manual, S. Gelvin and R. Schilperoort, eds., Kluwer Dordrecht, C1:1-19 (1994).

In some embodiments, an inducible transcription  
35 regulatory sequence can be coupled to a promoter sequence

- 18 -

functional in plants, both of which are operably linked to a polynucleotide of the invention. When such a regulatory element is coupled to a promoter, a truncated (or minimal) promoter generally is used, for example, the  
5 truncated 35S promoter of Cauliflower Mosaic Virus (CaMV). Truncated versions of other constitutive promoters can also be used, e.g., *A. tumefaciens* T-DNA genes such as *nos*, *ocs*, and *mas*, and plant virus genes such as the CaMV 19S gene.

10 Techniques are well-known to the art for the introduction of DNA into monocots as well as dicots, as are the techniques for culturing plant tissues and regenerating those tissues. Monocots which have been successfully transformed and regenerated include wheat,  
15 corn, rye, rice and asparagus. See, e.g., U.S. Patent Nos. 5,484,956 and 5,550,318. Transgenic aspen tissue has been prepared and transgenic plants have been regenerated. Poplars have also been transformed. Technology is also available for the manipulation,  
20 transformation, and regeneration of Gymnosperm plants. See, e.g., U.S. Patent No. 5,122,466 and U.S. Patent No. 5,041,382.

A method according to the invention includes the introduction of a nucleic acid construct into a plant  
25 cell and the production of a plant from such a transformed cell. Expression of the polynucleotide present in the construct alters the disease resistance phenotype of the plant, e.g., a novel disease resistance phenotype is conferred on the plant or an existing  
30 disease resistance phenotype is enhanced.

Disease resistance phenotype involves the level and timing of host defensive responses in the transgenic plant. Assays to indicate that disease resistance has been altered typically include the application of a  
35 compound that ordinarily elicits a defensive response to

- 19 -

a transgenic plant and, in parallel, the application of the same compound to a control plant. A control plant typically is from the same parental line as the one into which a new nucleic acid construct was introduced.

- 5 Disease resistance is enhanced or conferred on a plant by expression of a polynucleotide of the invention when there is a higher level of resistance in the transgenic plant than the corresponding resistance in the control plant. Disease resistance can be measured with reference  
10 to a specific pathogen, e.g., a *Phytophthora* spp.. Disease resistance can also be measured with reference to several pathogens, to identify an enhanced systemic defense response.

- Where transgenic plants are to be induced for  
15 expression of a CDPK coding sequence operably linked to an elicitor-mediated regulatory element, the elicitor typically must penetrate the cuticle of the plant to have an inductive effect. Plant tissue can be wounded to facilitate or allow the uptake of the elicitor into the  
20 plant tissue. A wide variety of inducing compositions, including elicitors and other chemical signals, such as the combination of ethylene and methyl jasmonate, can be effectively used to induce expression.

- A method of using a polynucleotide of the  
25 invention comprises the step of hybridizing the polynucleotide to DNA or RNA from a plant. Hybridization can be carried out, for example, as described hereinabove. The method can further comprise the step of identifying a segment of the plant DNA or RNA that has a  
30 significant degree of sequence identity to the polynucleotide, e.g., 70% sequence identity, preferably 80% sequence identity, 90% sequence identity, or 95% sequence identity. The segment can be identified by electrophoretic separation of the plant DNA or RNA and  
35 the use of labeled polynucleotide probe, which results in

- 20 -

a visible band at the position of the homologous segment. Segments can be generated, for example, by physical shearing or by restriction endonuclease digestion. A segment can be as short as 100 bp (nucleotides) in  
5 length, but typical segments are at least 1000 bp, and can be 10,000 bp or greater.

Such a method can further comprise the step of cloning at least a portion of the DNA or RNA segment, including, but not limited to, DNA flanking the  
10 homologous segment. Such flanking DNA can include promoters, enhancers, transcriptional regulatory elements and poly A sequences. Flanking DNA can be either 5' to or 3' to the homologous segment and preferably includes 300, or 600, or 1,000 bp of DNA beyond the coding  
15 sequence, because regulatory elements generally are found within this span.

Promoters and other elicitor or pathogen-responsive regulatory elements flanking the novel polynucleotides disclosed herein are particularly useful,  
20 because such elements confer very rapid induction of gene expression after treatment with pathogen or elicitor. Such regulatory elements can be operably linked to useful genes to allow rapid production of desirable compounds. For example, such regulatory elements can be used to  
25 drive expression of genes encoding antibodies, blood clotting factors, antigenic peptides, viral replicases or coat proteins, and enzymes involved in secondary metabolite synthesis (such as isoprenoid biosynthesis). See, e.g., U.S. Patent 5,612,487; U.S. Patent 5,484,719;  
30 and U.S. Application Ser. No. 08/577,483, filed December 22, 1995.

After introducing a chimeric gene having an elicitor or pathogen-responsive element into a plant, expression of the chimeric gene product can be induced  
35 with an appropriate pathogen or elicitor. Production of

- 21 -

the desired gene product (or its enzymatic end product) rapidly ensues and the desired product can then be obtained.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

#### EXAMPLES

The following examples use many techniques well-known and accessible to those skilled in the arts of molecular biology, in the manipulation of recombinant DNA in plant tissue and in the culture and regeneration of transgenic plants. Enzymes are obtained from commercial sources and are used according to the vendors' recommendations or other variations known to the art. Reagents, buffers, and culture conditions are also known to the art. Abbreviations and nomenclature, where employed, are deemed standard in the field and are commonly used in professional journals such as those cited herein.

20

#### Example 1.

##### Cloning of a Tobacco CDPK cDNA

The elicitor parasiticein was prepared by expression of the *Phytophthora parA1* gene in *E. coli* cells and isolation of the gene product from the periplasmic space.

Genomic DNA of *Phytophthora* Race O was isolated from mycelium essentially as described in Xu, J., et al. Trends in Genetics 10:226-227 (1994). The DNA was sheared and used as a template for PCR amplification of the *parA1* gene, using primers designed according to the *parA1* sequence reported in Kamoun, S., et al. Mol. Plant-Microbe Interact. 6:573-581 (1993). The *parA1* PCR product was cloned into pBluescript (Stratagene, San

- 22 -

Diego, CA) and the sequence of the product determined by double-stranded DNA sequencing using the dideoxy chain termination method.

The *parA1* insert in pBluescript was amplified by  
5 PCR, using primers that created an N-terminal histidine tag and a protein kinase site at the 5' end of the gene. The PCR product was ligated into the expression vector pET28b (Novagen, Madison, WI) and, after confirming the DNA sequence of the *parA1* fusion, the pET28b construct  
10 was transformed into *E. coli* BL21.

A BL21 culture containing the *parA1* fusion was grown at 37° C in the presence of kanamycin to an OD<sub>600</sub> of 0.3. IPTG (1mM) was added and the culture was incubated for 5 hours at 27° C.

15 Periplasmic proteins were prepared by osmotic shock essentially as described in Ausubel, F., et al. in Current Protocols in Molecular Biology, John Wiley & Sons, New York (1989). Cells (1.5 ml) were harvested by centrifugation, resuspended in 500 µl of 50 mM Tris-HCl,  
20 pH 8.0, 20% sucrose, 1 mM EDTA and incubated with shaking for 10 minutes at room temperature. After centrifugation, the pellet was resuspended in 200 µl ice cold MgSO<sub>4</sub> (5 mM) and incubated with shaking for 10 minutes at 4° C. The mixture was centrifuged and the  
25 resulting supernatant (containing periplasmic proteins) was applied to a Ni<sup>++</sup> column. The *parA1* protein was purified from the column according to the manufacturer's directions. The protein concentration in the *parA1* extract was determined by the Bradford method.

30 *Nicotiana tabacum* L. cv. KY14 cell suspension cultures were treated with parasiticein at a final concentration of 2 µg/ml during rapid growth phase to induce stress response genes. Parallel suspension cell cultures which were not treated with parasiticein served  
35 as controls. Cells were collected by gentle vacuum

- 23 -

filtration 0, 30, 60 and 120 minutes after the addition of elicitor.

Total RNA was isolated from treated and untreated tobacco cells and used as template for targeted differential display reverse transcriptase PCR (TDDRT-PCR). First strand cDNA was generated using a cDNA cycle kit from Invitrogen (San Diego, CA). The first strand cDNAs were then used as templates for PCR. The PCR reaction was carried out using typical conditions as described in PCR Protocols: A Guide to Methods and Applications, Innis, M., Gelfand, D., Sinsky, J. and White, T., eds. Academic Press Inc., San Diego, CA (1990), except that the annealing temperature was 58°C. The PCR primers were FokinB (GTTGACTCCCTACCCTCTT) and RecalIV (GGTACTTAGGAAGTGTACGGG). See Figure 1. PCR products were separated by electrophoresis on a 1% (w/v) agarose gel and products of greater than about 800 base pairs (bp) from the 60 minute treated culture were purified by electroelution onto DE-81 paper (Whatman). Ends of the purified PCR products were filled in with Klenow polymerase, ligated to the EcoRV site of pBluescript, and transformed into *E. coli* TB1.

Ampicillin resistant TB1 colonies were screened for the presence of a  $\geq 800$  bp DNA fragment inserted into pBluescript. The sequence of one such insert was determined by the dideoxynucleotide chain termination procedure of Sanger et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:8073-8077, with a Sequenase® kit from United States Biochemical Corp., Cleveland, OH) or an automated fluorescence based system (Applied Biosystems, Foster City, CA). The sequence of the insert in the vector was determined on both strands. The plasmid containing this insert was designated pCDPK-1.

The nucleotide sequence of the insert in pCDPK-1 is shown in Figure 2 and the deduced amino acid sequence

- 24 -

of the insert is shown in Figure 3. The deduced amino acid sequence was compared to amino acid sequences of plant genes in the GenBank, EMBL, and Swiss Prot databases. Homology was found to plant CDPK

5 polypeptides, including polypeptides from *Glycine max*, *Arabidopsis thaliana*, *Vigna radiata*, *Zea mays* and *Cucurbita pepo*.

Using the BLASTP program and a BLOSUM62 scoring matrix, two regions of homology to serine/threonine  
10 protein kinase domains were identified in the amino terminal portion of the polypeptide and four regions of homology to Ca<sup>++</sup> binding domains were identified in the carboxyl terminal portion of the polypeptide. Figure 4 shows a comparison of the amino acid sequence of Fig. 3  
15 and a soybean CDPK amino acid sequence (Genbank Accession No:M64987). The amino acid sequence of the tobacco calcium binding sites were similar to the amino acid sequence of corresponding sites in the soybean CDPK. However, there were significant differences in other  
20 parts of the sequence. The comparison indicates that there is about 78% overall sequence identity between the soybean CDPK and CDPK-1.

The BLASTN program was used to compare the pCDPK-1 nucleotide sequence to nucleic acid sequences on various  
25 databases. Based on the nucleotide sequence of other plant CDPK genes and the length of the polypeptides encoded thereby, the nucleic acid insert present in pCDPK-1 is estimated to lack about 560 bp of 5' CDPK-1 coding sequence and about 130 bp of 3' CDPK-1 coding  
30 sequence.

#### Example 2.

##### Isolation of a full-length cDNA clone

To obtain a full-length clone, a RACE (Rapid Amplification of cDNA Ends) approach is used, with polyA+  
35 RNA prepared from tobacco cells after induction with

- 25 -

elicitor being the template. PolyA+ RNA is prepared as described in Example 1.

A primer having the sequence GAC AAG GAC GGG AGT GGG TAT (Primer A, internal to CDPK-1) and a primer  
5 having the sequence GAC TCG AGT CGA CAT CGA TTT TTT TTT TTT TTT TT (dT<sub>17</sub> adapter-primer) are used to amplify the 3' end of the CDPK coding sequence. The reverse transcriptase reaction is carried out in 2 µl 10X RTC buffer, 10 units of RNasin (Promega Biotech), 0.5 µg of  
10 dT<sub>17</sub> adapter-primer and 10 Units of AMV reverse transcriptase (Life Sciences) in a total volume of 3.5 µl, as described in Frohman, M. in PCR Protocols: A Guide to Methods and Applications, *supra*, pp. 28-38. The PCR amplification reaction is carried out in 5 µl 10X PCR  
15 buffer, 5 µl DMSO, 5 µl 10X dNTPs (15 mM each), 30 µl H<sub>2</sub>O, 1 µl adapter-primer (25 pmol, GAC TCG AGT CGA CAT CG), 1 µl primer A and 1-5µl cDNA. Cycle times are as indicated in Frohman, *supra*.

The 5' end of the CDPK coding sequence is cloned  
20 by carrying out reverse transcription as described above, using 10 pmole of primer B (AGG GGC TAC GTA GTA AGG ACT) instead of dT<sub>17</sub> adapter-primer. The cDNA product is extended using terminal transferase and dATP as described in Frohman, *supra*, and then amplified by PCR as described  
25 above with 10 pmole of dT<sub>17</sub> adapter-primer, 10 pmole of adapter-primer and 10 pmole of primer C (ATT CTC AGG CTT AAG GTC CCT). PCR is carried out under standard conditions. Back et al. (1994) *Arch. Biochem. Biophys.* 315:523-532. The amplified 3' and 5' products are blunt-  
30 end cloned into pBluescript SK (Stratagene) and combined with the pCDPK-1 insert by routine molecular biology techniques to form a full-length cDNA of the tobacco CDPK coding sequence.

- 26 -

The DNA sequence of the full-length cDNA is determined by a dideoxynucleotide chain termination procedure, as described in Example 1.

### Example 3.

#### 5                   Induction of CDPK-Homologous RNA                   in Tobacco Suspension Cultures

The DNA insert in pCDPK-1 was used as a probe to follow the induction of gene expression in response to elicitor. *Nicotiana tabacum* L. cv. KY14 cell suspension  
10 cultures were treated with parasiticein for 0, ½, 1, 2, 6 and 12 hours as described in Example 1. Total RNA was isolated and electrophoresed on a 1% agarose gel. The insert from pCDPK-1 was radiolabeled by the random priming method and hybridized to the gel-separated RNA as  
15 described in Sambrook, J. et al., *supra*. No mRNA hybridizing to CDPK-1 was detected prior to elicitor treatment, whereas mRNA hybridizing to CDPK-1 was readily detected at 1/2, 1 and 2 hours after elicitor treatment. At 6 and 12 hours after elicitor treatment, no mRNA  
20 hybridizing to CDPK-1 could be detected, indicating that CDPK-1 gene expression had decreased to undetectable levels by about 6 hours.

### Example 4.

#### Construction of a Chimeric CDPK Gene

25           A CDPK gene is constructed from: a chemically synthesized DNA encoding amino acids 1 to 156 of the soybean CDPK of Figure 6, a chemically synthesized DNA encoding amino acids 465 to 508 of the soybean CDPK of Figure 6, and the CDPK insert of pCDPK-1. The three DNAs  
30 are ligated by routine molecular biology techniques to form a chimeric CDPK coding sequence having amino acids 1 to 156 of soybean CDPK at the amino terminal end, fused

- 27 -

in-frame to amino acids 1 to 307 of tobacco CDPK (Fig. 3), which in turn is fused in-frame to amino acids 465 to 508 of soybean CDPK at the carboxyl terminal end.

The chimeric coding sequence is inserted in sense orientation into an *Agrobacterium* binary vector containing a minimal 35S and EAS4 inducible regulatory element. Operable linkage of the regulatory element, promoter, and coding sequence is confirmed by determining the DNA sequence of the junction regions and by expression in transgenic plants.

#### Example 5.

##### Generation of Transgenic Plants

Transformed plant cell lines are produced using a modified *Agrobacterium tumefaciens* transformation protocol. Nucleic acid constructs are prepared that contain the full-length CDPK cDNA of Example 3 or the chimeric CDPK coding sequence of Example 4. The recombinant constructs containing the sequences to be introduced into plants are transferred into *A. tumefaciens* strain GV3850 by triparental mating with *E. coli* TB1 (pRK2013). *N. tabacum* leaves at a variety of stages of growth are cut into 1 cm<sup>2</sup> pieces, and dipped in a suspension of *Agrobacterium* cells (about 10<sup>4</sup> to 10<sup>5</sup> cells/ml). After 3 to 10 minutes, the leaf segments are then washed in sterile water to remove excess bacterial cells and to reduce problems with excess bacterial growth on the treated leaf segments. After a short drying time (30 to 60 seconds), the treated leaf segments are placed on the surface of Plant Tissue Culture Medium without antibiotics to promote tissue infection and DNA transfer from the bacteria to the plant tissue. Plant Tissue Culture Medium contains per liter: 4.31 g of Murashige and Skoog Basal Salts Mixture (Sigma Chemical Company, St. Louis, MO), 2.5 mg of benzylaminopurine (dissolved in

- 28 -

1 N NaOH), 10 ml of 0.1 mg/ml indoleacetic acid solution, 30 g sucrose, 2 ml of Gamborg's Vitamin Solution (Sigma Chemical Co., St. Louis, MO) and 8 g of agar. The pH is adjusted between pH 5.5 and 5.9 with NaOH. After 2 days, the leaf segments are transferred to Plant Tissue Culture Medium containing 300  $\mu$ g/ml of kanamycin, 500  $\mu$ g/ml of mefoxin (Merck, Rahway, NJ). Kanamycin selects for transformed plant tissue, and mefoxin selects against *Agrobacterium*.

It may be necessary to minimize the exposure of the explant tissue to *Agrobacterium* cells during the transformation procedure if a pathogen-inducible regulating element is used, because *Agrobacterium* cells may themselves induce the element after introduction into the plant cells. Accordingly, the biolistic technique for the introduction of DNA containing cell suicide genes under the regulatory control of the inducible transcriptional regulatory element is a useful alternative transformation technique because it does not entail the use of *Agrobacterium* cells or fungal cell wall digestive enzymes (as necessary for the generation of protoplasts for electroporation), both of which can lead to induction of the coding sequences under the control of that regulatory element.

Transgenic plants are regenerated essentially as described by Horsch et al. (1985) *Science* 227:1229-1231.

#### Example 6.

##### Elicitor- and Pathogen-inducible Expression of a Chimeric CDPK Gene in Transgenic Tobacco

The activity of the CDPK constructs of Example 7 are measured in transgenic tobacco plants treated with either an elicitor or pathogen. As controls, transgenic tobacco plants expressing the GUS reporter gene under the control of the cauliflower mosaic virus (CaMV) 35S

- 29 -

promoter are also produced. F<sub>1</sub> seeds from regenerated transgenic tobacco plants are germinated on medium containing 100 mg/L kanamycin. The resulting kanamycin-resistant plants are subsequently transferred  
5 into soil and grown in a greenhouse. Half of the plants are tested for the expression of the CDPK gene under inducing conditions, e.g., by intercellular application of elicitor or cellulase to the transgenic plants. Elicitor or cellulase is applied with a mechanical  
10 pipetter. As a control, remaining plants are mock-treated with a solution lacking cellulase or elicitor. Tobacco tissue is wounded with a scalpel in some experiments to facilitate exposure to the inducing compound.

15

**Example 7.****Identification of CDPK Homologous Sequences**

Tobacco leaf genomic DNA is isolated as described in Murray and Thompson (1980) *Nucleic Acids Research* 8:4321-4325. After digestion of aliquots with desired  
20 restriction enzymes, the digested DNA samples are electrophoresed on 0.8% agarose gels and the size-separated DNAs are transferred to nylon membranes. DNA blots are hybridized with the 900 bp CDPK cDNA insert of Example 1 that is radiolabeled by the random primer  
25 method. Hybridization is performed at 60°C in 0.25 M sodium phosphate buffer, pH 8.0, 0.7% SDS, 1% bovine serum albumin, 1 mM EDTA. The blot is then washed twice at 45°C with 2X SSC, 0.1% SDS and twice with 0.2X SSC, 0.1% SDS (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate,  
30 pH 7.0). Relative hybridization intensities of the various bands on the membrane are estimated from autoradiograms using a video densitometer (MilliGen/Biosearch, Ann Arbor, MI).

- 30 -

To identify polynucleotides having homologous sequences to tobacco CDPK and to determine the apparent number of copies per genome of those sequences, Southern hybridization experiments are carried out using target DNA isolated from other plant species and tobacco CDPK probes. Restriction endonuclease-digested genomic DNAs of various plant species are separated by agarose gel electrophoresis (0.8% agarose), and then transferred to a Hybond-N<sup>+</sup> membrane (Amersham Corp., Arlington Heights, IL). Radiolabeled probe fragments comprising coding sequences of pCDPK-1 are hybridized to the digested genomic DNA essentially as described in Sambrook et al. (1989), supra. Moderate stringency conditions are used (hybridization in 4X SSC, at 65°C with the last wash in 1X SSC, at 65°C).

Alternatively, PCR is carried out using target genomic DNA as a template and primers derived from highly conserved regions of the pCDPK-1 coding sequence.

#### Example 8.

##### Genomic DNA Flanking a CDPK Coding Sequence

The cDNA clone described in Example 1 is used as a hybridization probe for screening a *N. tabacum* cv. NK326 genomic library in the  $\lambda$ EMBL3 vector (Clontech, Palo Alto, CA). Genomic DNA clones having 70% or greater sequence identity to the tobacco CDPK of Example 1 are identified using routine subcloning protocols. The nucleotide sequences of the cloned nucleic acid inserts are determined using routine DNA sequencing protocols.

One of the genomic DNA clones has a full-length coding sequence that comprises the tobacco CDPK coding sequence of Example 1. The clone also contains DNA contiguous with, and 5' to, the coding sequence of Example 1. Examination of the nucleotide sequence of the 5' flanking DNA in this clone reveals a putative ATG

- 31 -

start codon as well as one or more putative regulatory elements upstream of the start codon and within about 1000 bp of the start codon.

Other Embodiments

5           It is to be understood that while the invention has been described in conjunction with the Detailed Description thereof, that the foregoing description is intended to illustrate, and not limit the scope of the invention, which is defined by the scope of the appended  
10 claims. Other aspects, advantages, and modifications are within the scope of the following claims.

- 32 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT: University of Kentucky Research Foundation
- (ii) TITLE OF THE INVENTION: PROTEIN KINASES AND USES THEREOF
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Fish & Richardson P.C., P.A.
  - (B) STREET: 60 South Sixth Street, Suite 3300
  - (C) CITY: Minneapolis
  - (D) STATE: MN
  - (E) COUNTRY: USA
  - (F) ZIP: 55402
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: FastSEQ for Windows Version 2.0
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE: 07-JUL-1998
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: 08/889,655
  - (B) FILING DATE: 08-JUL-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Lundquist, Ronald C
  - (B) REGISTRATION NUMBER: 37,875
  - (C) REFERENCE/DOCKET NUMBER: 07678/020W01
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 612-335-5050
  - (B) TELEFAX: 612-288-9696
  - (C) TELEX:

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 921 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

AGGGACCTTA AGCCTGAGAA TTTCCTTTTC AGTGCCGACG ACTTCATGGT AAAGAGTAAG 60
GCCACCGACT TCGGGCTTAG TGTATTCTAT AAGCCTGGGC AAAAGTTCAC GGACATAGTA 120
GGGAGTCCTT ACTACGTAGC CCCTGAGGTA CTTAGGAAGT GTTACGGGCC TGGGAGTGAC 180
GTATGGAGTG CCGGGGTAAT ACTTTACACC CTTCTTTGTG GGGCCCCCTCC TTTCATGGCC 240
GACAGTGAGC CTGGGGTAGC CCTTCAAATA CTTTCATGGG ACCTTGACTT CAAGAGTGAC 300
CCTTGGCCTA CCATAAGTGA GAGTGCCAAG GACCTTATAA GGAAGATGCT TGAGCAAGAC 360
CCTAAGAGGA GGCTTACCGC CCATGAGGTA CTTAGGCATC CTTGGATAGT AGACGAGAAT 420

```

- 33 -

```

ATAGCCCCCTG ACAAGCCTCT TGGGCCTGCC GTACTTAGTA GGCTTAAGCA ATTCAGTGCC 480
ATGAATAAGA TAAAGAAGAT GGCCCTTAGG GTAATAGCCG AGAGGCTTAG TGAGGAGGAG 540
ATAGTAGGGC TTAAGGAGAT GTTCAAGATG GACACCGACA ATAGTGGGAC CGTAACCTTC 600
TTCCATCTTA AGCAAGGGCT TAAGAGGGTA GGGAGTCAAC TTGGGGAGAG TGAGATAAAG 660
GACCTTATGG ACGCCGCCGA CGTAGACAAT AGTGGGACCA TAGACTATGG GGAGTTCGTA 720
ACCGCCGCCA TGCATCTTAA TAAGATAAAG AGGGAGGACC ATCTTGTAAG TGCCTTCAGT 780
TATCATGACA AGGACGGGAG TGGGTATATA GAGGTAGACG AGCTTAGGCA AGCCCTTGAG 840
GAGTTCGGGG TACCTGACAC CAGTCTTGAG GACATGATAA AGGAGGTAGA CACCGACAAT 900
GATGGGCAAA TAGATTATGG G                                     921

```

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Arg Asp Leu Lys Pro Glu Asn Phe Leu Phe Ser Ala Asp Asp Phe Met
 1          5          10          15
Val Lys Ser Lys Ala Thr Asp Phe Gly Leu Ser Val Phe Tyr Lys Pro
 20          25          30
Gly Gln Lys Phe Thr Asp Ile Val Gly Ser Pro Tyr Tyr Val Ala Pro
 35          40          45
Glu Val Leu Arg Lys Cys Tyr Gly Pro Gly Ser Asp Val Trp Ser Ala
 50          55          60
Gly Val Ile Leu Tyr Thr Leu Leu Cys Gly Ala Pro Pro Phe Met Ala
 65          70          75          80
Asp Ser Glu Pro Gly Val Ala Leu Gln Ile Leu His Gly Asp Leu Asp
 85          90          95
Phe Lys Ser Asp Trp Pro Thr Ile Ser Glu Ser Ala Lys Asp Leu
100          105          110
Ile Arg Lys Met Leu Glu Gln Asp Pro Lys Arg Arg Leu Thr Ala His
115          120          125
Glu Val Leu Arg His Pro Trp Ile Val Asp Glu Asn Ile Ala Pro Asp
130          135          140
Lys Pro Leu Gly Pro Ala Val Leu Ser Arg Leu Lys Gln Phe Ser Ala
145          150          155          160
Met Asn Lys Ile Lys Lys Met Ala Leu Arg Val Ile Ala Glu Arg Leu
165          170          175
Ser Glu Glu Glu Ile Val Gly Leu Lys Glu Met Phe Lys Met Asp Thr
180          185          190
Asp Asn Ser Gly Thr Val Thr Phe Phe His Leu Lys Gln Gly Leu Lys
195          200          205
Arg Val Gly Ser Gln Leu Gly Glu Ser Glu Ile Lys Asp Leu Met Asp
210          215          220
Ala Ala Asp Val Asp Asn Ser Gly Thr Ile Asp Tyr Gly Glu Phe Val
225          230          235          240
Thr Ala Ala Met His Leu Asn Lys Ile Lys Arg Glu Asp His Leu Val
245          250          255
Ser Ala Phe Ser Tyr His Asp Lys Asp Gly Ser Gly Tyr Ile Glu Val
260          265          270
Asp Glu Leu Arg Gln Ala Leu Glu Glu Phe Gly Val Pro Asp Thr Ser
275          280          285
Leu Glu Asp Met Ile Lys Glu Val Asp Thr Asp Asn Asp Gly Gln Ile
290          295          300
Asp Tyr Gly
305

```

- 34 -

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTACTTAGG AAGTGTTACG GG

22

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTGACTCCC TACCCTCTT

19

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 512 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```

Met Ala Ala Lys Ser Ser Ser Ser Thr Thr Thr Asn Val Val Thr
 1          5          10          15
Leu Lys Ala Ala Trp Val Leu Pro Gln Arg Thr Gln Asn Ile Arg Glu
 20          25          30
Val Tyr Glu Val Gly Arg Lys Leu Gly Gln Gly Gln Phe Gly Thr Thr
 35          40          45
Phe Glu Cys Thr Arg Arg Ala Ser Gly Gly Lys Phe Ala Cys Lys Ser
 50          55          60
Ile Pro Lys Arg Lys Leu Leu Cys Lys Glu Asp Tyr Glu Asp Val Trp
 65          70          75          80
Arg Glu Ile Gln Ile Met His His Leu Ser Glu His Ala Asn Val Val
 85          90          95
Arg Ile Glu Gly Thr Tyr Glu Asp Ser Thr Ala Val His Leu Val Met
100          105          110
Glu Leu Cys Glu Gly Gly Glu Leu Phe Asp Arg Ile Val Gln Lys Gly
115          120          125
His Tyr Ser Glu Arg Gln Ala Ala Arg Leu Ile Lys Thr Ile Val Glu
130          135          140
Val Val Glu Ala Cys His Ser Leu Gly Val Met His Arg Asp Leu Lys
145          150          155          160
Pro Glu Asn Phe Leu Phe Asp Thr Ile Asp Glu Asp Ala Lys Leu Lys
165          170          175
Ala Thr Asp Phe Gly Leu Ser Val Phe Tyr Lys Pro Gly Glu Ser Phe
180          185          190
Cys Asp Val Val Gly Ser Pro Tyr Tyr Val Ala Pro Glu Val Leu Arg
195          200          205
Lys Leu Tyr Gly Pro Glu Ser Asp Val Trp Ser Ala Gly Val Ile Leu

```

- 35 -

210		215		220
Tyr	Ile	Leu	Leu	Ser
225		230		235
Gly	Ile	Phe	Arg	Gln
		245		250
Pro	Trp	Pro	Ser	Ile
		260		265
Leu	Asp	Gln	Asn	Pro
		275		280
His	Pro	Trp	Ile	Val
		290		295
Ser	Ala	Val	Leu	Ser
305		310		315
Lys	Lys	Met	Ala	Leu
		325		330
Ile	Gly	Gly	Leu	Lys
		340		345
Gly	Thr	Ile	Thr	Phe
		355		360
Lys	Arg	Val	Gly	Ser
		370		375
Asp	Ala	Ala	Asp	Ile
		385		390
Ile	Ala	Ala	Thr	Val
		405		410
Val	Ser	Ala	Phe	Ser
		420		425
Leu	Asp	Glu	Ile	Gln
		435		440
His	Ile	Asp	Asp	Met
		450		455
Ile	Asp	Tyr	Gly	Glu
		465		470
Ile	Gly	Arg	Arg	Thr
		485		490
Gly	Leu	Val	Asp	Asn
		500		505
				510

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GACAAGGACG GGAGTGGGTA T

21

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACTCGAGTC GACATCGATT TTTTTTTTTT TTTT

35

(2) INFORMATION FOR SEQ ID NO:8:

- 36 -

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GACTCGAGTC GACATCG

17

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AGGGGCTACG TAGTAAGGAC T

21

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATTCTCAGGC TTAAGGTCCC T

21

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 308 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Arg Asp Leu Lys Pro Glu Asn Phe Leu Phe Ser Ala Asp Asp Phe Met
 1           5           10           15
Val Lys Ser Lys Ala Thr Asp Phe Gly Leu Ser Val Phe Tyr Lys Pro
          20           25           30
Gly Gln Lys Phe Thr Asp Ile Val Gly Ser Pro Tyr Tyr Val Ala Pro
          35           40           45
Glu Val Leu Arg Lys Cys Tyr Gly Pro Gly Ser Asp Val Trp Ser Ala
          50           55           60
Gly Val Ile Leu Tyr Thr Leu Leu Cys Gly Ala Pro Pro Phe Met Ala
          65           70           75           80
Asp Ser Glu Pro Gly Val Ala Leu Gln Ile Leu His Gly Asp Leu Asp
          85           90           95
Phe Lys Ser Asp Pro Trp Pro Thr Ile Ser Glu Ser Ala Lys Asp Leu
          100          105          110
Ile Arg Lys Met Leu Glu Gln Asp Pro Lys Arg Arg Leu Thr Ala His
          115          120          125
Glu Val Leu Arg His Pro Trp Ile Val Asp Glu Asn Ile Ala Pro Asp

```

- 37 -

130		135		140
Lys Pro Leu Gly Pro Ala Val Leu Ser Arg Leu Lys Gln Phe Ser Ala				
145		150		155
Met Asn Lys Ile Lys Lys Met Ala Leu Arg Val Ile Ala Glu Arg Leu				160
	165		170	175
Ser Glu Glu Glu Ile Val Gly Leu Lys Glu Met Phe Lys Met Ile Asp				
	180		185	190
Thr Asp Asn Ser Gly Thr Val Thr Phe Phe His Leu Lys Asp Gly Leu				
	195		200	205
Lys Arg Val Gly Ser Gln Leu Gly Glu Ser Glu Ile Lys Asp Leu Met				
	210		215	220
Asp Ala Ala Asp Val Asp Asn Ser Gly Thr Ile Asp Tyr Gly Glu Phe				
225		230		235
Val Thr Ala Ala Met His Leu Asn Lys Ile Lys Arg Glu Asp His Leu				240
	245		250	255
Val Ser Ala Phe Ser Tyr His Asp Lys Asp Gly Ser Gly Tyr Ile Glu				
	260		265	270
Val Asp Glu Ile Arg Gln Ala Leu Glu Glu Phe Gly Val Pro Asp Thr				
	275		280	285
Ser Leu Glu Asp Met Ile Lys Glu Val Asp Thr Asp Asn Asp Gly Gln				
	290		295	300
Ile Asp Tyr Gly				
305				

- 38 -

## WHAT IS CLAIMED IS:

1. An isolated polynucleotide, said polynucleotide comprising:
  - a) the nucleotide sequence of SEQ ID NO:1;
  - 5 b) an RNA analog of SEQ ID NO:1;
  - c) a polynucleotide comprising a nucleic acid sequence complementary to a) or b); or
  - d) a nucleic acid fragment of a), b) or c) that is at least 20 nucleotides in length and that hybridizes
  - 10 under stringent conditions to genomic DNA encoding the polypeptide of Figure 3.
2. The polynucleotide of claim 1, wherein said polynucleotide comprises nucleotides 1 to 170 of Figure 2.
- 15 3. The polynucleotide of claim 1, wherein said polynucleotide comprises nucleotides 160 to 560 of Figure 2.
4. The polynucleotide of claim 1, wherein said polynucleotide comprises nucleotides 550 to 920 of Figure
- 20 2.
5. A nucleic acid construct comprising the polynucleotide of claim 1.
6. The nucleic acid construct of claim 5, further comprising a regulatory element operably linked
- 25 to said polynucleotide.
7. The nucleic acid construct of claim 6, wherein said regulatory element is an inducible regulatory element.

- 39 -

8. The nucleic acid construct of claim 7,  
wherein said regulatory element is induced in response to  
a plant pathogen.

9. A transgenic plant containing a nucleic acid  
5 construct comprising the polynucleotide of claim 1.

10. The plant of claim 9, wherein said construct  
further comprises a regulatory element operably linked to  
said polynucleotide.

11. The plant of claim 10, wherein said  
10 regulatory element is an inducible regulatory element.

12. The plant of claim 11, wherein said  
regulatory element is induced in response to a plant  
pathogen.

13. The plant of claim 11, wherein said  
15 regulatory element is induced in response to an elicitor.

14. The plant of claim 9, wherein said plant is a  
dicotyledonous plant.

15. The plant of claim 14, wherein said plant is  
a member of the *Solanaceae* family.

20 16. The plant of claim 15, wherein said plant is  
a *Nicotiana* plant.

17. The plant of claim 16, wherein said plant is  
*Nicotiana tabacum*.

18. A transgenic plant containing a  
25 polynucleotide expressing a polypeptide having from about

- 40 -

250 to about 550 amino acids, said polypeptide comprising an amino acid sequence substantially identical to the amino acid sequence of Figure 3.

19. The plant of claim 18, wherein said  
5 polypeptide comprises the amino acid sequence of Figure 3.

20. The plant of claim 18, wherein said plant is a dicotyledonous plant.

21. The plant of claim 20, wherein said plant is  
10 a member of the *Solanaceae* family.

22. A method of using a polynucleotide, said method comprising the step of hybridizing the polynucleotide of claim 1 to DNA or RNA from a plant.

23. The method of claim 22, further comprising  
15 the step of identifying a segment of said plant DNA or RNA that has about 70% or greater sequence identity to said polynucleotide.

24. The method of claim 23, further comprising the step of cloning at least a portion of said DNA or RNA  
20 segment.

25. The method of claim 24, wherein said cloned portion further comprises DNA flanking said segment having 70% or greater sequence identity.

26. A method of altering disease resistance in a  
25 plant, said method comprising the steps of:

(a) introducing the nucleic acid construct of claim 5 into a plant cell; and

- 41 -

(b) producing a plant containing said polynucleotide from said cell, wherein expression of said polynucleotide alters disease resistance in said plant.

27. The method of claim 26, wherein said nucleic  
5 acid construct further comprises an inducible regulatory element operably linked to said polynucleotide and said expression is regulated by said regulatory element.

28. The method of claim 27, wherein said  
expression is induced by said regulatory element upon  
10 exposure of said plant to an elicitor or plant pathogen.

29. An isolated polypeptide having from about 250  
to about 550 amino acids, said polypeptide comprising an  
amino acid sequence substantially identical to Figure 3.

30. The polypeptide of claim 29, wherein said  
15 polypeptide comprises the amino acid sequence of Figure  
3.

# FIGURE 1

## Primers

RecalV - 1 = GTTGACTCCCTACCCCTCTT  
↓  
CALCIUM BINDING SITE

FokinB - 1 = GGTACTTAGGAAGTGTTACGGG  
↓  
KINASE DIAGNOSTIC SEQUENCE

## FIGURE 2A

```

      10      20      30      40
      *      *      *      *
AGG GAC CTT AAG CCT GAG AAT TTC CTT TTC AGT GCC GAC GAC TTC
TCC CTG GAA TTC GGA CTC TTA AAG GAA AAG TCA CGG CTG CTG AAG

      50      60      70      80      90
      *      *      *      *      *
ATG GTA AAG AGT AAG GCC ACC GAC TTC GGG CTT AGT GTA TTC TAT
TAC CAT TTC TCA TTC CGG TGG CTG AAG CCC GAA TCA CAT AAG ATA

      100     110     120     130
      *      *      *      *
AAG CCT GGG CAA AAG TTC ACG GAC ATA GTA GGG AGT CCT TAC TAC
TTC GGA CCC GTT TTC AAG TGC CTG TAT CAT CCC TCA GGA ATG ATG

      140     150     160     170     180
      *      *      *      *      *
GTA GCC CCT GAG GTA CTT AGG AAG TGT TAC GGG CCT GGG AGT GAC
CAT CGG GGA CTC CAT GAA TCC TTC ACA ATG CCC GGA CCC TCA CTG

      190     200     210     220
      *      *      *      *
GTA TGG AGT GCC GGG GTA ATA CTT TAC ACC CTT CTT TGT GGG GCC
CAT ACC TCA CGG CCC CAT TAT GAA ATG TGG GAA GAA ACA CCC CGG

      230     240     250     260     270
      *      *      *      *      *
CCT CCT TTC ATG GCC GAC AGT GAG CCT GGG GTA GCC CTT CAA ATA
GGA GGA AAG TAC CGG CTG TCA CTC GGA CCC CAT CGG GAA GTT TAT

      280     290     300     310
      *      *      *      *
CTT CAT GGG GAC CTT GAC TTC AAG AGT GAC CCT TGG CCT ACC ATA
GAA GTA CCC CTG GAA CTG AAG TTC TCA CTG GGA ACC GGA TGG TAT

      320     330     340     350     360
      *      *      *      *      *
AGT GAG AGT GCC AAG GAC CTT ATA AGG AAG ATG CTT GAG CAA GAC
TCA CTC TCA CGG TTC CTG GAA TAT TCC TTC TAC GAA CTC GTT CTG

      370     380     390     400
      *      *      *      *
CCT AAG AGG AGG CTT ACC GCC CAT GAG GTA CTT AGG CAT CCT TGG
GGA TTC TCC TCC GAA TGG CGG GTA CTC CAT GAA TCC GTA GGA ACC

      410     420     430     440     450
      *      *      *      *      *
ATA GTA GAC GAG AAT ATA GCC CCT GAC AAG CCT CTT GGG CCT GCC
TAT CAT CTG CTC TTA TAT CGG GGA CTG TTC GGA GAA CCC GGA CGG

      460     470     480     490
      *      *      *      *
GTA CTT AGT AGG CTT AAG CAA TTC AGT GCC ATG AAT AAG ATA AAG
CAT GAA TCA TCC GAA TTC GTT AAG TCA CGG TAC TTA TTC TAT TTC

      500     510     520     530     540
      *      *      *      *      *

```

## FIGURE 2B

```

AAG ATG GCC CTT AGG GTA ATA GCC GAG AGG CTT AGT GAG GAG GAG
TTC TAC CGG GAA TCC CAT TAT CGG CTC TCC GAA TCA CTC CTC CTC

      550      560      570      580
      *      *      *      *
ATA GTA GGG CTT AAG GAG ATG TTC AAG ATG GAC ACC GAC AAT AGT
TAT CAT CCC GAA TTC CTC TAC AAG TTC TAC CTG TGG CTG TTA TCA

      590      600      610      620      630
      *      *      *      *      *
GGG ACC GTA ACC TTC TTC CAT CTT AAG CAA GGG CTT AAG AGG GTA
CCC TGG CAT TGG AAG AAG GTA GAA TTC GTT CCC GAA TTC TCC CAT

      640      650      660      670
      *      *      *      *
GGG AGT CAA CTT GGG GAG AGT GAG ATA AAG GAC CTT ATG GAC GCC
CCC TCA GTT GAA CCC CTC TCA CTC TAT TTC CTG GAA TAC CTG CGG

      680      690      700      710      720
      *      *      *      *      *
GCC GAC GTA GAC AAT AGT GGG ACC ATA GAC TAT GGG GAG TTC GTA
CGG CTG CAT CTG TTA TCA CCC TGG TAT CTG ATA CCC CTC AAG CAT

      730      740      750      760
      *      *      *      *
ACC GCC GCC ATG CAT CTT AAT AAG ATA AAG AGG GAG GAC CAT CTT
TGG CGG CGG TAC GTA GAA TTA TTC TAT TTC TCC CTC CTG GTA GAA

      770      780      790      800      810
      *      *      *      *      *
GTA AGT GCC TTC AGT TAT CAT GAC AAG GAC GGG AGT GGG TAT ATA
CAT TCA CGG AAG TCA ATA GTA CTG TTC CTG CCC TCA CCC ATA TAT

      820      830      840      850
      *      *      *      *
GAG GTA GAC GAG CTT AGG CAA GCC CTT GAG GAG TTC GGG GTA CCT
CTC CAT CTG CTC GAA TCC GTT CGG GAA CTC CTC AAG CCC CAT GGA

      860      870      880      890      900
      *      *      *      *      *
GAC ACC AGT CTT GAG GAC ATG ATA AAG GAG GTA GAC ACC GAC AAT
CTG TGG TCA GAA CTC CTG TAC TAT TTC CTC CAT CTG TGG CTG TTA

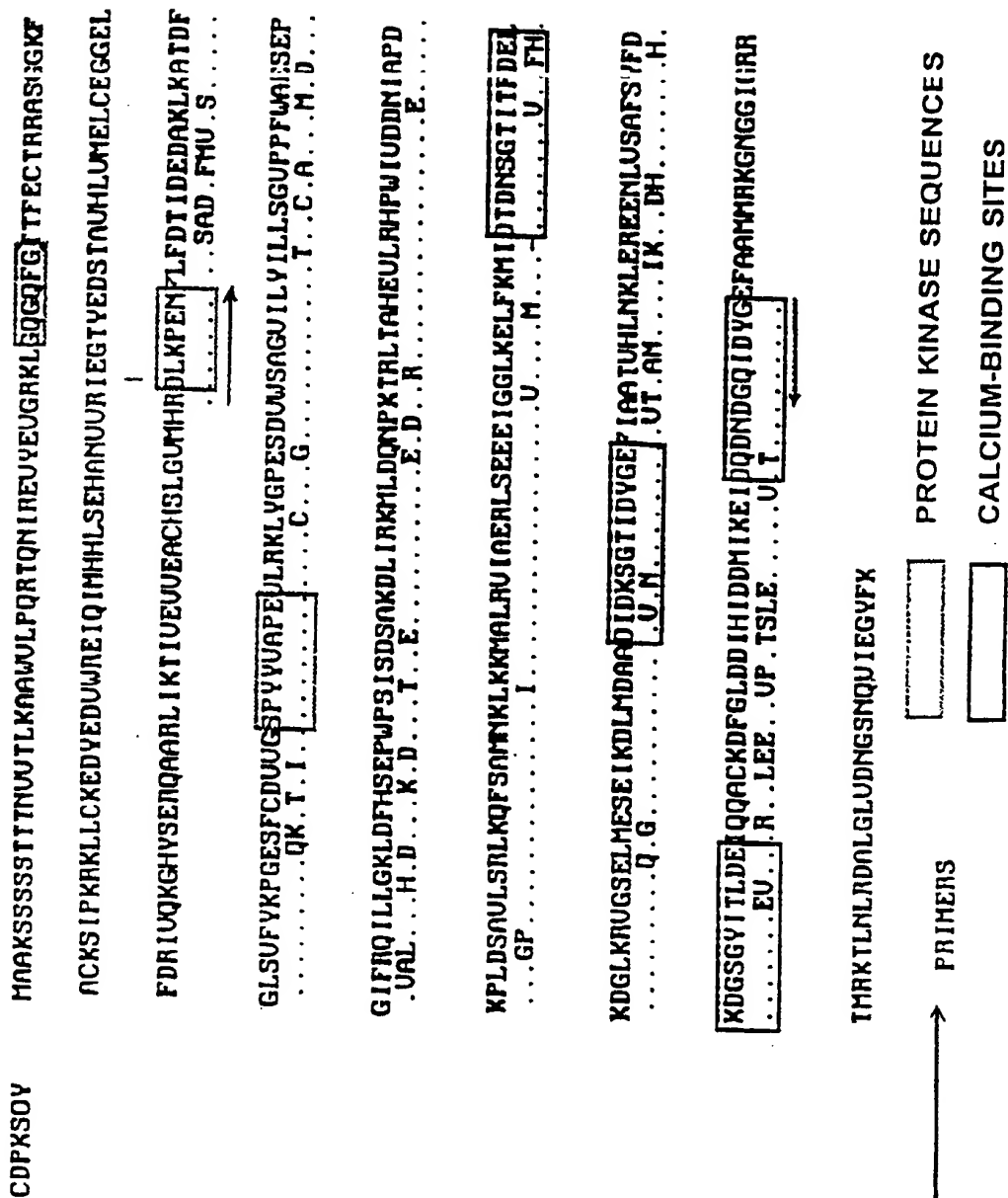
      910      920
      *      *
GAT GGG CAA ATA GAT TAT GGG
CTA CCC GTT TAT CTA ATA CCC

```

## FIGURE 3

10	20	30	40	
*	*	*	*	
RDL KPE NEL TBA DDF MKR SKA TDF GLE VTY KRG QMT TDI VGS PYY				
50	60	70	80	90
*	*	*	*	*
VAP EVL RKC YGP GSD VWS AGV ILY TLL CGA PPF MAD SEP GVA LQI				
100	110	120	130	
*	*	*	*	
LHG DLD FKS DPW PTI SES AKO LIR KML EQD PKR RLT AHE VLR HPW				
140	150	160	170	180
*	*	*	*	*
IVD ENI APD KPL GPR VLS RLK QFS AMN KIK KMA LRV IAE RLS EEE				
190	200	210	220	
*	*	*	*	
IVG LKE MEK MDT DNS GTV TFF HLK QGL KRV GSQ LGE SEI KDL MDA				
230	240	250	260	270
*	*	*	*	*
ADV DNS GTI DYG EFV TAA MHL NKI KRE DHL VSA FSY HDX DGS GYI				
280	290	300		
*	*	*		
EVD ELR QAL EEF GVP DTS LED MIK EVD TDN DGQ IDY G				

# FIGURE 4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/14109

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C12N 5/04, 5/10, 5/16, 5/22, 15/82; A01H 5/00

US CL : 435/34; 800/278,530/300

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/34; 800/278,530/300

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS. MEDLINE, BIOSIS, AGRICOLA

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y  Y	HARPER et al. A Calcium-Dependent Protein Kinase with a Regulatory Domain Similar to Calmodulin. Science. 17 May 1991, Vol. 252, page 951-954, see entire document.  VALVEKENS et al. Agrobacterium Tumefaciens-Mediated Transformation of Arabidopsis Thaliana Root Explants by Using Kanamycin Selection. Proc. Natl. Acad. Sci. August 1988, Vol. 85, pages 5536-5540, see entire document.	1 _____ 2-21, 26-28  2-21,26-28

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

02 OCTOBER 1998

Date of mailing of the international search report

23 OCT 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

OUSAMA M-FAIZ ZAGHMOUT

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/14109

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-21,26-28

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/14109

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I. Claims 1-21 and 26-28 are drawn to nucleic acid molecule encoding CDPK from tobacco, vectors containing it in sense orientation, methods for their use to transform plants, and the resultant plants.

Group II. Claims 22-25 are drawn to the use of polynucleotides to isolate DNA fragment comprising less than full length gene.

Group III. Claims 29-30 are drawn to CDPK protein.

The inventions listed as groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Since fragment of nucleotide sequence of the protein is known in the art as evidenced by the Harper et al reference (Science. 1991. Vol. 252:951-954), it does not constitute a special technical feature as defined by PCT Rule 13.2. Groups I-III are directed to isolation and use of nucleic acid from plant cells and their expression in transgenic plants in addition to the CDPK protein of group III. However, since claim 1 lacks novelty, unity of invention is lacking, because fragment of nucleotide sequence of the protein was reported previously by the Harper et al reference (Science. 1991. Vol. 252:951-954). The cited evidence proves that the technical feature of group I, fragment of nucleotide sequence of the protein, does not make a contribution over the prior art. The claims are not so linked by a special technical feature within the meaning of the PCT Rule 13.2 so as to form a single inventive concept, accordingly, the unity of invention is lacking among all groups.